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Fitness and pathogenicity of the fungi associated with the mountain pine beetle and other secondary beetles in green attack

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Abstract

The mountain pine beetle (MPB) (*Dendroctonus ponderosae*) and its fungal associates kill healthy lodgepole pine forests in western Canada and the north-western United States. In previous work we characterized the fungal associates of MPB and analyzed their phylogenetic relationships. In this work we re-assessed the identification of a few species, including *O. nicrocarpum*-like (now *O. abietinum*-like), the genera *Graphium* and *Ambrosiella*, and the *O. minutum*-like isolates. We also assessed the pathogenicity of five isolates of *O. clavigerum* and re-examined the virulence indicators for fungal pathogens. Finally, we determined the physiological characteristics of a few strains of *O. clavigerum*. While the work on fungal identity is not totally completed, good progress has been made and further work will be pursued in 2007-2008. We are now confident that the fungi identified as *Ambrosiella* sp. were in fact *Ophiostoma* species. The description of the epitype for *O. minutum* will be completed soon; the fungi originally described as *O. minutum*-like are more closely related to *Ophiostoma manitobense*. One of the MPB-associates, *O. clavigerum*, is pathogenic and can kill trees when inoculated in the absence of the beetle. Consequently, field studies were initiated to examine variation in virulence indicators (lesion length, moisture content and occlusion area) for five strains of *O. clavigerum*. These studies were necessary since only one isolate has been tested in this epidemic and because population analyses of over 100 isolates have shown different grouping of the isolates. Significant differences between strains were observed for all indicators measured. The strain SL-kW 1407, which was used most recently for testing the pathogenicity of *O. clavigerum* in the field, was found to be the least virulent among the five fungal strains, indicating *O. clavigerum* may be even more virulent than previously thought. Finally, we assessed the same five strains for the growth response to temperature variation, terpene treatment, oxygen deficiency, lipids and pH. Optimal growth was found between 22.5 to 25°C and at pH 5.5-6.0. The isolates showed reduced growth when exposed to terpenes, linoleic acid and coconut oil. The growth was inhibited by capric and myristic acids. The growth was slightly increased under anaerobic environment. Characterizing the pathogenicity and physiology of the fungi (e.g., *O. clavigerum*) contributes to the understanding of the MBP outbreak.

Keywords: Fungal identification and phylogeny; *O. nicrocarpum*-like, *O. minutum*-like, genera *Graphium* and *Ambrosiella*, pathogenicity, growth.

Résumé

Le dendroctone du pin ponderosa (DPP) (*Dendroctonus ponderosae*) et ses associés fongiques dévastent des forêts de pins tordus en santé dans l'Ouest canadien et le nord-ouest des États-Unis. Nous avons déjà caractérisé les associés fongiques du DDP et analysé leurs relations phylogéniques. Dans la présente étude, nous avons réévalué la présence de quelques espèces, dont les espèces de la famille *O. nicrocarpum* (maintenant appelée *O. abietinum*), les genres *Graphium* et *Ambrosiella*, et les isolats de la famille *O. minutum*. Nous avons également évalué la pathogénicité de cinq isolats d'*O. clavigerum*

et réexaminé les indicateurs de virulence pour les pathogènes fongiques. Enfin, nous avons déterminé les caractéristiques physiologiques de quelques souches d'*O. clavigerum*. Bien que les travaux d'identification des champignons ne soient pas terminés, nous avons réalisé des progrès importants et les travaux se poursuivront en 2007-2008. Nous reconnaissons maintenant que les champignons identifiés comme étant de l'*Ambrosiella* sp. sont en fait des espèces d'*Ophiostoma*. La description de l'épitype de l'*O. minutum* sera bientôt terminée. Les champignons initialement décrits comme faisant partie de la famille de l'*O. minutum*, sont plus étroitement apparentés à l'*Ophiostoma manitobense*. Un des associés du DPP, l'*O. clavigerum*, est un pathogène capable de tuer des arbres lorsqu'il est inoculé, en absence de dendroctone. Par conséquent, des études sur le terrain ont été entreprises afin d'examiner la variation des indicateurs de virulence (longueur des lésions, teneur en humidité et zone d'occlusion) de cinq souches d'*O. clavigerum*. Ces études étaient nécessaires puisqu'un seul isolat avait été mis à l'essai dans la présente infestation et parce que les analyses de la population de plus de 100 autres isolats ont montré un regroupement des isolats différent. On a observé des différences importantes entre les souches dans le cas de tous les indicateurs mesurés. La souche SL-kW 1407, qui a été la dernière à être utilisée pour éprouver la pathogénicité d'*O. clavigerum* sur le terrain, s'est révélée la moins virulente des cinq souches fongiques, ce qui indique qu'*O. clavigerum* pourrait être plus virulent qu'on ne le croyait. Pour terminer, nous avons analysé les cinq mêmes souches afin de déterminer leur croissance en fonction d'un changement de température, leur traitement terpène, l'insuffisance d'oxygène, les lipides et le pH. La croissance maximale a été observée entre 22,5 et 25°C et à un pH se situant entre 5,5 et 6,0. Nous avons observé une diminution de la croissance des isolats lorsqu'ils étaient exposés à des terpènes, à de l'acide linoléique et à de l'huile de noix de coco. L'acide décanoïque et l'acide tétradécanoïque ont freiné la croissance. La croissance a légèrement augmenté dans un environnement anaérobique. La caractérisation de la pathogénicité et de la physiologie des champignons (p. ex., *O. clavigerum*) aide à mieux comprendre l'infestation de DPP.

Mots-clés : Identification fongique et phylogenèse; famille *O. nicrocarpum*, famille *O. minutum*, genres *Graphium* et *Ambrosiella*, pathogénicité, croissance.

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1 Introduction

The mountain pine beetle (MPB), *Dendroctonus ponderosae* Hopkins, is the most destructive bark beetle in western Canada. In the past decade, more than 9.0 million hectares of lodgepole pines have been killed (British Columbia Ministry of Forests 2007). MPB is associated with several species of fungi that aid the beetle in successfully attacking and colonizing trees. The majority of these fungal species belong to the Ophiostomatoid group of fungi. Diversity of fungi involved in the current mountain pine beetle epidemic in British Columbia have been extensively studied by Kim et al. (2005), Lim et al. (2005) and Lee et al. (2006a). *Ophiostoma clavigerum* (Robinson-Jeffrey & Davidson) Harrington, *O. montium* (Rumbold) von Arx and *Ceratocystiopsis minutum* (Olchow & Reid) Hausner, Reid & Klassen are the most common species found in these studies. This *C. minutum* has been reported as *O. minutum*-like. It is a slow-growing non-sapstaining fungus that is poorly characterized in the literature. The taxonomy of this species is discussed in more detail below. The presence of other Ophiostomatoid fungi including the newly-described species *Leptographium longiclavatum* S. Lee, J.-J. Kim & C. Breuil (Lee et al. 2005), *Ophiostoma nigrocarpum* (Davidson) De Hoog-like, as well as the undescribed species of the genera *Graphium* and *Ambrosiella* are also reported (Kim et al., 2005). Preliminary identifications were re-assessed and new or tentative identifications are further discussed in this report.

Ophiostoma clavigerum is a highly virulent sapstain fungus that is capable of killing trees in the absence of the beetle (Lee et al., 2006); however, very little is known about intraspecific variation in virulence for this species. The objectives of these experiments were to characterize the intraspecific variation in virulence indicators of *O. clavigerum*, and to compare two time periods for growth inside trees in relation to virulence indicators. To the author's knowledge, this multi-strain pathogenicity testing is the first to be done for any fungal associate of the mountain pine beetle and will be only the third paper to examine multi-strain pathogenicity on any bark beetle-fungal associate system in the field. It is also the first time a comparison has been attempted between significantly different time periods of incubation.

In this report we also reported some preliminary results on the growth on artificial media of the five strains of *O. clavigerum* used in field tests. We examined their optimal temperature and pH for growth and the effects of fatty acids, terpenes and oxygen on the growth of the different strains.

2 Materials and Methods

2.1 Morphological and molecular characteristics of the unknown species.

The morphological characteristics and the sequences analyses of different isolates were re-examined. Some of the re-assessed isolates are listed in Table 1.

Table 1: Identification of the MPB-associated fungi

Code	Host	Substrate	Origin	Preliminary ID[1]	Final ID
872AW	Lodgepole pine	Wood infested by MPB[2]	Canada/BC	*Ambrosiella* sp.	*Ophiostoma* sp.
878AW1-1	Lodgepole pine	Wood infested by MPB	Canada/BC	*Ambrosiella* sp.	*Ophiostoma* sp.
873AW1-2	Lodgepole pine	Wood infested by MPB	Canada/BC	*Ambrosiella* sp.	*Ophiostoma* sp.
877EW2-1	Lodgepole pine	Wood infested by MPB	Canada/BC	*Ambrosiella* sp.	Needs further study
841EW 2-1	Lodgepole pine	Wood infested by MPB	Canada/BC	*Graphium* sp. 3	*Graphium* sp. 3
841EW 2-2	Lodgepole pine	Wood infested by MPB	Canada/BC	*Graphium* sp. 3	*Graphium* sp. 3
MPBON-1	Lodgepole pine	Wood infested by MPB	Canada/BC	*Ophiostoma nigrocarpum*-like	*Ophiostoma abietinum*
SL-A54	Lodgepole pine	Mountain Pine Beetle	Canada/BC	*Ophiostoma nigrocarpum*-like	*Ophiostoma abietinum*

1 : Kim et al. 2005 ; 2 : Mountain Pine Beetle (*Dendroctonus rufipennis*)

2.2 The phylogeny of the *O. mintum* species complex

In order to properly characterize the '*O. minutum*' found on the MPB, three strains from the MPB, 10 strains from other global locations, and seven strains from Poland were assembled, along with one to three strains of many other closely related species. The protein coding Beta-tubulin region and two regions from rDNA, the ITS and LSU areas, were sequenced and phylogenetic analysis was conducted using maximum parsimony, maximum likelihood and neighbour-joining analysis. Special attention was paid to strains from Poland since the original holotype sample, which was lost during World War 2, came from Poland. Morphological measurements are ongoing for several of these strains in order to determine a proper epitype strain and to properly characterize the MPB-associated '*O. minutum*.'

2.3 Variation in virulence indicators of *O. clavigerum*

In order to assess the intraspecific variation in virulence indicators, five strains of *O. clavigerum* plus sterile agar controls were inoculated at a density of 200 holes/m^2 into 20-year old lodgepole pines. The inoculated trees were left for 11 months in the field ("Year 1 testing"). Harvested trees were transported back to the lab and the virulence indicators: lesion length, moisture content and occlusion area were measured. During the first year, tree family background was accounted for, but since this did not significantly affect results, family background was ignored the second year. The five fungal strains plus controls were inoculated into 21-year old lodgepole pines during the second year and left to grow for 2 months in the field ("Year 2 testing"). The same virulence indicators

were measured and within- and between-years variations were compared. Two fungal strains, one deemed 'virulent' and one 'less virulent,' based on first year results, were inoculated into 10 mature trees, ~80 years old, to determine whether results from younger trees were comparable to older trees. Mature trees are more likely to be attacked by the MPB.

A simulated in vitro experiment was also conducted in which the five fungal strains plus controls were inoculated into freshly cut logs of mature lodgepole pines and incubated in the lab for 2 months in order to assess the reproducibility of the in vivo experiments in an 'easier to conduct' setting ("*in vitro* testing").

2.4 Physiological characteristics of five *O. clavigerum* strains

The five *O. clavigerum* strains used in the experiments described below were the same strains used in pathogenicity. They were ATCC18086 [holotype], B5, B20, H55 and SL-Kw1407.

For growth at different temperatures, fungal plugs for each of the five strains of *O. clavigerum* were placed in the middle of 2% OMEA agar plates. For each temperature, three replicates per strain were used and the entire experiment was replicated once. Plates were incubated in the dark for 4 days. Each plate was measured in two different locations to check for consistent growth. Temperatures tested were 4°C, 10°C, 15°C, 20°C, 22.5°C, 25°C, 27.5°C, 30°C and 37°C.

For growth at different pHs (4.0 to 8.0), 2% MEA was adjusted to the appropriate pH in 0.5 pH increments. Inoculation and growth measurements were done as described above.

For growth in presence of terpenes, approximately 200 µL of terpenes were sprayed on 2% OMEA plates. Fungal plugs for each of five *O. clavigerum* strains were placed in the middle of the treated 2% OMEA agar plates. Controls were 2% OMEA plate with no terpene treatment.

For growth in an anaerobic environment, fungal plugs for each of five *O. clavigerum* strains were placed in the middle of 2% OMEA agar plates. Two small pieces of tygon tubing (~2-4 cm) were placed on the sides of petri dishes to allow for gas movement. Petri dishes were placed inside large glass bells that were sealed with silicon vacuum grease. Oxygen was replaced by pumping in pure nitrogen into the headspace of the bell. Plates were incubated in the dark for 4 days. Two measurements per plate were made and the experiment was replicated three times. Controls were performed under atmospheric oxygen conditions.

For growth with capric (C10:0), myristic (C14:0), palmitic (C16:0), stearic (C18:0), oleic (C18:1), linoleic (C18:2), glycerol, olive and coconut oils, the lipids were dissolved in 0.1% tergitol (detergent) before being added to the YNB agar media containing 0.3% asparagine as a nitrogen source. Inoculation and growth measurements were as described above.

3 Results and Discussion

3.1 Reassessing the identity of a few species using morphological and molecular characteristics

Morphological and molecular characteristics of the undescribed species from the current MPB epidemics (Table 1) have been further studied to confirm their preliminary identifications. Results show that strains identified as unknown species of the genus *Graphium* Corda are synnematous-forming fungi that are clustered together with other species of *Graphium* in a highly supported monophyletic clade within Microascales (Figs.1 and 3). This is consistent with Okada et al. (2000), describing the anamorph genus *Graphium*, which confirm the preliminary identification of the synnematous-forming associates of MPB as new species of the genus *Graphium*.

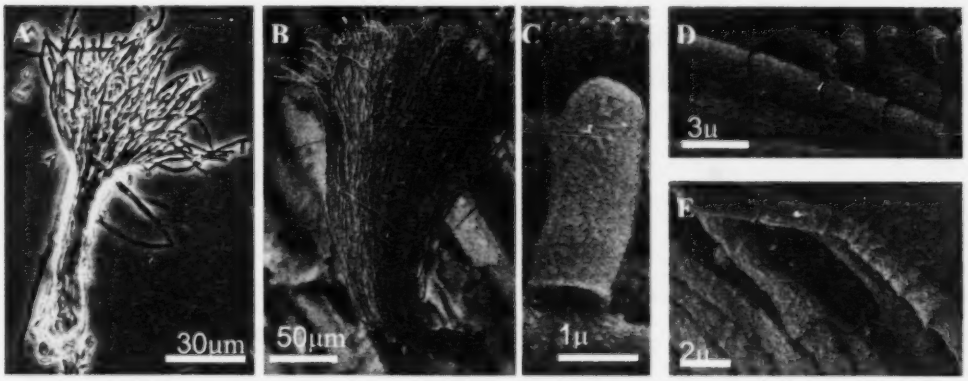

Figure 1. *Graphium* associate of MPB. **A-B**. Synnemata with stipe of parallel hyphae and divergent capitulum. **C.** Conidia with conspicuous basal frills. **D.** Conidia occurring in short chains. **E.** Conidiogenous cells with pronounced annellations.

Morphological and molecular observations of the fungal strains identified as undescribed species of the genus *Ambrosiella* by Kim et al. (2005), however, do not support the genus identified. Originally, *Ambrosiella* has been described to accommodate the primary associate of ambrosia beetle *A. xylebori* (Brader 1964). Later, Batra (1967) included nine additional species to this genus that resemble the type species of *Ambrosiella*, in lacking a known sexual state and producing monilioid conidiophores loosely arranged in a confluent sporodochia (Brader 1964; Batra 1967).

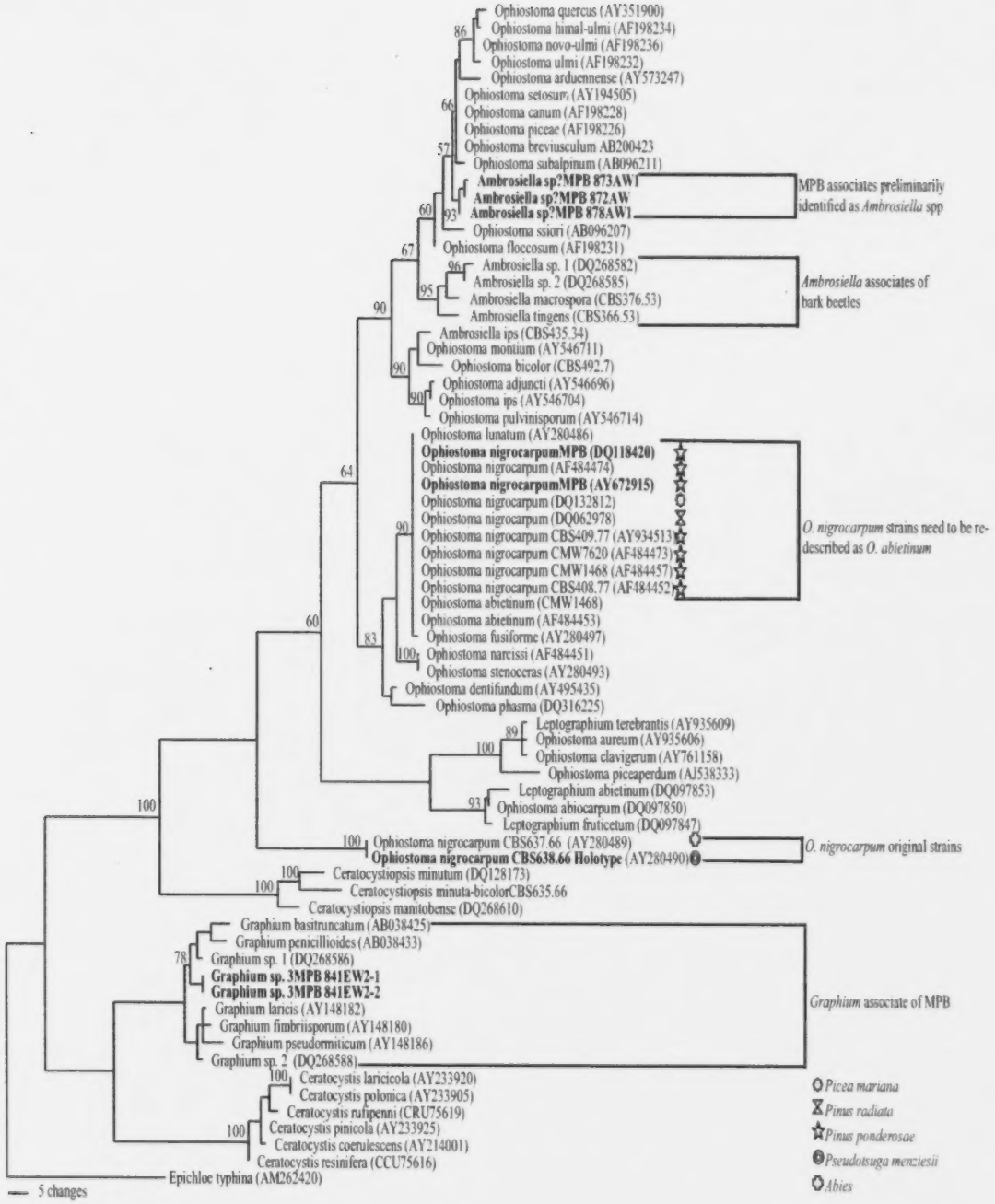

Figure 2. One of six most parsimonious trees showing phylogenetic relationships between the MPB-associates and other known species of ophiostomatoid fungi. The tree was generated from the heuristic analysis of ribosomal DNA (ITS2) sequence data. Bootstrap values (1000 replicates) greater than 50% are indicated at the branch nodes. Fungal taxa collected and identified during this work are given in bold.

Of these species, seven are mutualistic symbionts of wood-boring beetles and are accepted as true ambrosia, whereas three, *A. macrospora* (Francke-Grosmann) Batra; *A. tingens* (Lagerberg & Melin) Batra and *A. ips* (Leach, Orr & Chrisensen) Batra (Fig. 3), are bark beetle-associated species and have not been recognized as true ambrosia associates (Funk 1970; Batra 1967). However, in the taxonomic revision of the hitherto described ambrosia fungi, Batra (1967), based on morphology, amended the three bark-beetle associates to the genus *Ambrosiella.*

Characteristics of the unknown MPB associates preliminary identified as *Ambrosiella* do not fit the morphological concept of the genus *Ambrosiella*: monilioid conidiophores and the confluent Sporodochia. Furthermore, although their rDNA showed the highest sequence identity with those of *A. macrospora* (Kim et al. 2005), they form monophyletic clades separated from the *Ambrosiella* associates of both bark (Fig. 3) and ambrosia beetles (data not shown). The β-tubulin phylogenetic tree suggests (Fig. 2) that these MPB associates are two phylogenetically unrelated groups. The first group (Figs. 1-2) produces perithecia (sexual structure of Ophiostomatoid fungi), forms a monophyletic clade closely related to the *Piceae*-complex with 98% bootstrap support, and therefore, should be re-described as a new species of the genus *Ophiostoma.*

The second group shows (Fig. 2) the closest relationship to an *Ambrosiella* associate of bark beetles *A. ips*. Morphological observations of isolates from the second group indicate that these fungi lack stable, well-defined characteristics. Further, *A. ips* is the sister taxon of *O. montium*; both species share similar morphological and molecular characteristics. For example, the numbers and arrangements of β-tubulin introns in *O. montium* are very different from those of other *Ophiostoma* species, but they are similar to those found in *A. ips* and the unknown MPB-associate. Thus, studies are needed to re-assess the taxonomic positioning of *A. ips* and to clarify whether this fungus and the unknown MPB-associate should be re-described as part of a complex including *O. montium.*

Finally, the identity of the species described by Kim et al.(2005) as *O. nigrocarpum* was re-assessed. Originally, the description was based on a specimen received from Aghayeva et al. (2004) and labelled *O. nigrocarpum*. However, the isolate received was improperly labelled *O. nigrocarpum*. The β-tubulin of the authentic *O. nigrocarpum* (Davidson) de Hoog was sequenced and compared to the MPB *O. nigrocarpum* isolates. The MPB isolates were more closely related to that of the ex-type isolate of *O. abietinum* (De Beer et al. 2003) than to *O. nigrocarpum* and were renamed *O. abietinum*-like.

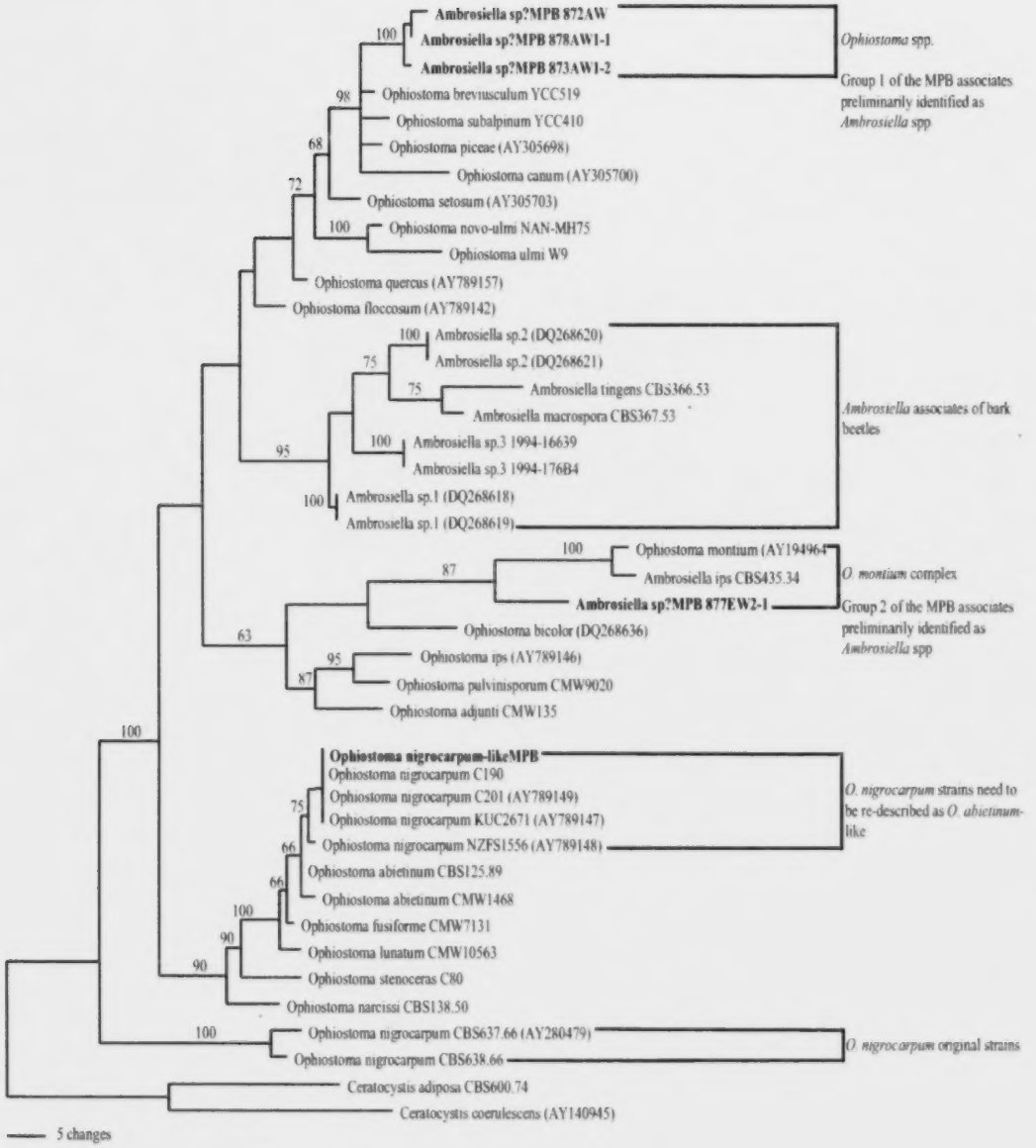

Figure 3. One of three most parsimonious trees showing phylogenetic relationships between the MPB-associates and other known species of ophiostomatoid fungi. The tree was generated from the heuristic analysis of β-tubulin exon sequence data. Bootstrap values (1000 replicates) greater than 50% are indicated at the branch nodes. Fungal taxa collected and identified during this work are given in bold. *Ceratocystis adiposa* and *Ceratocystis coerulescens* were used as outgroup taxa to root the phylogenetic tree.

3.2 The phylogeny of the *O. minutum* species complex

The MPB-associated '*Ophiostoma minutum*' is genetically distinct from all other strains of *O. minutum*. As such, it will be referred to as '*O. minutum*-like' until we can give it a proper name. We have selected a tentative epitype strain from among Polish isolates based on DNA sequences (see Figure 4) and are awaiting morphological characters to develop in order to properly characterize the potential epitype strain. This is a slow growing species and fruiting bodies take upwards of 3 months to form. We have also identified several errors in previous literature stemming from confusion over what exactly is *O. minutum*. DNA sequences suggest these strains have been misnamed. Once perithecia develop and we can properly characterize '*O. minutum*,' we will also characterize the new *O. minutum*-like strain found on mountain pine beetles.

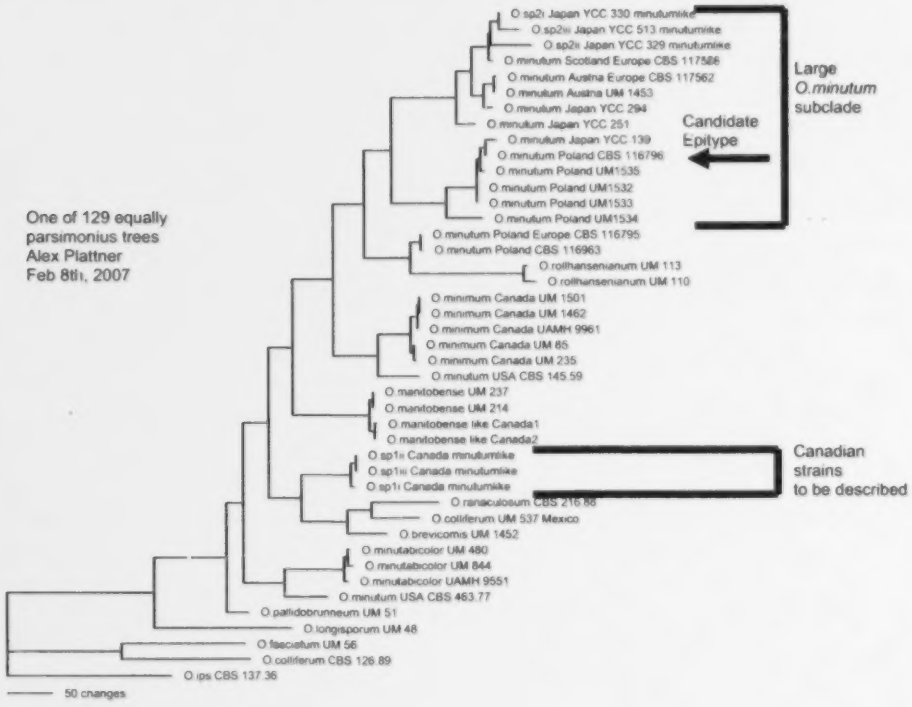

Figure 4. One of 129 equally parsimonious trees showing a candidate epitype strain for *Ophiostoma minutum* and the position of the genetically distinct *Ophiostoma minutum*-like strains found on mountain pine beetle infested wood.

3.3 Variation in virulence indicators of *O. clavigerum*

Results from Year 1

Statistically, fast and slow growing trees did not differ significantly in terms of moisture content and occlusion area, and only sometimes in terms of lesion length. As such, results were pooled and testing done the following year did not take tree family background into account. During the first year, the strains ATCC 18086 (type culture), B5 and H55 had more pronounced indicators of pathogenicity than strains KW 1407 or B20 (see Figure 5).

Results from Year 2

During the second year, strain ATCC 18086 had more pronounced indicators of pathogenicity compared to all other strains (see Figure 6). Results from the same year follow the same pattern regardless of which pathogenicity indicator is used. In other words, if a strain produces longer lesions, it also tends to reduce the moisture content in the tree and occlude a larger area of the sapwood. Results from different years suggest that shorter time periods do not allow for symptoms to fully develop, further suggesting that a longer time of incubation with the pathogen is more useful when measuring virulence indicators.

Results from mature trees

The results from inoculating the virulent and less virulent strain into mature trees suggest that patterns of virulence indicators seen in younger trees are similar to that of older trees. Although there was significant variation among trees, as is expected, in no case was the average lesion length for the less virulent strain longer than the average lesion length for the virulent strain.

Results from in vivo testing

Thirty freshly cut logs were inoculated with the five strains of *O. clavigerum* in order to assess the ability of in vitro results to mimic in vivo results. Results suggest in vitro testing does not simulate natural conditions well enough and is not a reliable method of testing pathogenicity. Although lesion length, the only pathogenicity indicator measured, produced somewhat similar results to field tests, the actual growth of the fungus did not appear to be characteristic of what occurs in live trees and as such, we do not recommend this type of testing for future use.

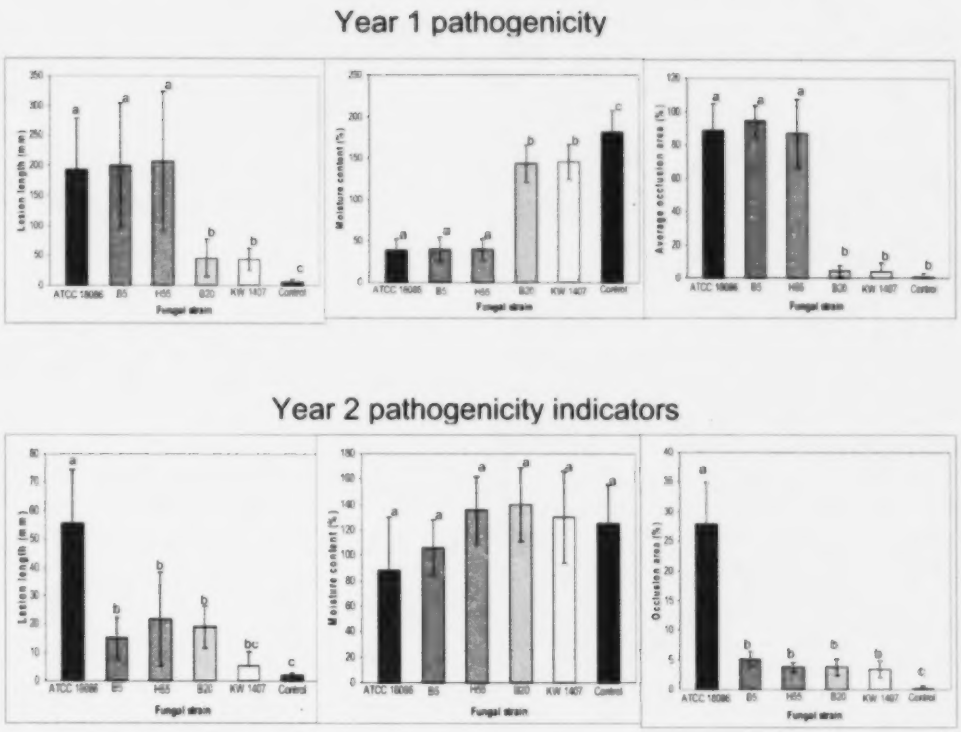

Figure 5. Year 1 (top) and year 2 (bottom) pathogenicity indicators from left to right: lesion length, moisture content and occlusion area. Bars with the same letter are not significantly different from each other at a 0.05 level. Strains ATCC 18086, B5 and H55 consistently produced more pronounced virulence measures compared to strains B20, KW 1407 and controls during the first year. Strain ATCC 18086 produced more pronounced virulence measures compared to all other strains during the second year.

3.4 Physiological characteristics of a few *O. clavigerum* strains

Temperature dependent growth

Strain ATCC 18086 grew the fastest at lower temperatures but grew slowest at 27.5. No strains grew well at 30°C (maximum growth at 5 days was 4 mm) and no strains grew at all at 37°C (Fig. 6, left side)

Growth in presence of terpenes

Strain ATCC 18086 also showed the largest percentage reduction in growth when grown on a blend of major terpenes from lodgepole pine oleoresin, although the absolute growth rate was still fastest (see Figure 6, right side). Overall, the average reduction in growth from the presence of terpenes was ~one-third.

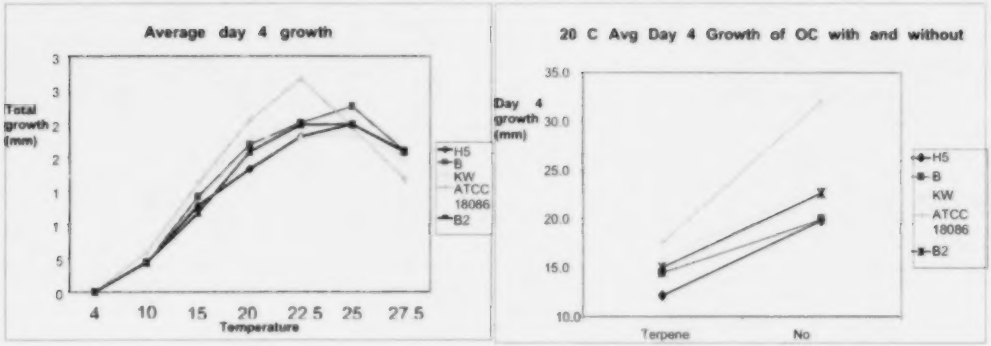

Figure 6. Average growth after 4 days at various temperatures (left) and day 4 growth at 20°C with and without anti-fungal metabolites (right) for five strains of *Ophiostoma clavigerum*. Strain ATCC 18086 grew the fastest at lower temperatures but experienced the largest reduction of growth at higher temperatures and had the largest percentage reduction in growth in the presence of the blend of major terpenes from lodgepole pine oleoresin. All strains experienced roughly a one-third drop off in growth rate when grown on the blend of terpenes from lodgepole pine oleoresin.

Growth with oxygen deficiency

Strains grown in oxygen deficient conditions grew faster than the same strains grown in the presence of oxygen. The sapwood of trees contains very low oxygen levels; as such, the ability to grow under oxygen deficient conditions is a key characteristic of primary sapwood invaders. At 20°C, the five fungal strains tested had significantly different growth rates ($p < 0.0001$). The absence of oxygen had a significant effect on growth rate ($p<0.0001$); strains grew better in the absence of oxygen (average growth = 26.1 mm) than in the presence of oxygen (average growth = 20.6 mm) after 4 days. The presence of terpenes significantly reduced the growth of all fungal strains ($p < 0.0001$). In the presence of terpenes, strains grew an average of 18.8 mm after four days, whereas without terpenes, strains grew an average of 27.9 mm. All fungal strains were equally affected by the absence of oxygen ($p = 0.15$), whereas the fungal strains reacted differently to the presence of terpenes ($p < 0.0001$). The fastest growing strain, ATCC 18086, experienced a reduction in growth of ~15 mm, whereas all other strains experienced a reduction in growth of ~7-8 mm.

Effects of lipids on fungal growth

The results showed that three fatty acids (capric, myristic, and linoleic acids) and coconut oil had an inhibitory effect on all the fungal strains. Capric acid was antifungal at concentrations as low as 0.004%. However, the ATCC strain eventually grows at 0.008%. Capric acid has been reported to affect H(+)-ATPase activity (Alexandre et al. 1996). Coconut oil was also inhibitory, likely due to its constituents (1.0% capric and 2.0% lauric acid). Myristic acid, another fungal inhibitor (Uziel and Kenneth 1999) was less toxic on *O. clavigerum* than capric acid. Linoleic acid at 1% had only a slightly

inhibitory effect on the growth. We are currently testing the nitrogen requirement of these five strains.

4 Conclusions

1. *Graphium* species associated with MPB are new species that need to be further characterized. The *Ambrosiella* sp. described originally seem to have been misidentified and have been described as unknown *Ophiostoma* sp. Further identification work is needed for these species. *O. nigrocarpum*-like were re-identified as *O. abietinum*-like.

2. The phylogeny of the *O. minutum* species complex is presented and the description of the new epitype (strain CBS 116796) is ongoing. Genetic evidence indicated that strains of '*O. minutum*-like' associated with the MPB belong to a separate species. A new name for this species will be provided in a future publication.

3 Variation in virulence and significant intraspecific variation exists among strains of *O. clavigerum*. A period of growth longer than 2 months is recommended for future tests.

5 Strains of *O. clavigerum* grow best at 22.5°C – 25°C in accordance with previous literature results, and are negatively impacted by the presence of lodgepole pine terpenes and fatty acids. The absence of oxygen appears to slightly increase the growth rate of all strains of *O. clavigerum*, although more work needs to be carried out to confirm these preliminary results.

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6 References

Aghayeva DN, Wingfield MJ, De Beer ZW, Kirisits T (2004). Two new *Ophiostoma* species with *Sporothrix* anamorphs from Austria and Azerbaijan. Mycologia 96: 866-878.

Alexandre, H., Mathieu, B. and Charpentier, C. 1996. Alteration in membrane fluidity and lipid composition, and modulation of H(+)-ATPase activity in *Saccharomyces cerevisiae* caused by decanoic acid. Microbiology.142 (Pt3):469-475.

Batra, L.R. (1967) Ambrosia fungi: a taxonomic revision and nutritional studies of some species. Mycologia 59: 976–1017.

Brader, L. (1964) Etude de la relation entre le scolyte des rameaux du cafe´ ier, Xyleborus compactus Eichh. (X. morstatti Hag.), et sa plante-ho ˆ te. Mededelingen van de Landbouwhogeschool Wageningen 64: 1–109.

British Columbia Ministry of Forests. Information Bulletin Feb. 17, 2007. 9.2 Million hectares affected by mountain pine beetle. 2007FOR0011-000152.

De Beer, Z.W., Harrington, T.C., Vismer, H.F., Wingfield, B.D., Wingfield, M.J. (2003). Phylogeny of the *Ophiostoma stenoceras–Sporothrix schenckii* complex. Mycologia 95: 434–441.

Funk, A. (1970) Fungal symbionts of the ambrosia beetle *Gnathotrichus sulcatus*. Canadian Journal of Botany 48:1445–1448.

Kim, J.J., E.A. Allen, L.M. Humble and C. Breuil. 2005. Ophiostomatoid and basidiomycetous fungi associated with green, red, and grey lodgepole pines after mountain pine beetle (*Dendroctonus ponderosae*) infestation. Canadian Journal of Forest Research 35: 274-284.

Lee, S., J. Kim and C. Breuil (2005). *Leptographium longiclavatum sp. nov.*, a new species associated with the mountain pine beetle, *Dendroctonus ponderosae*. Mycological. Research. 109: 275-284.

Lee, S., J.-J. Kim, and C. Breuil 2006a. Fungal diversity associated with the mountain pine beetle, *Dendroctonus ponderosae* and infested lodgepole pines in British Columbia. Fungal Diversity 22:91-105.

Lee, S., J.J., Kim and C. Breuil 2006b "Pathogenicity of *Leptographium longiclavatum* associated with *Dendroctonus ponderosae* to Pinus contorta" Canadian Journal of Forest Research 36: 2864-2872.

Lee, S., R.C. Hamelin, D.L. Six and C. Breuil. 2007. Genetic diversity and the presence of two distinct groups in *Ophiostoma clavigerum* associated with *Dendroctonus ponderosae* in British Columbia and the northern Rocky Mountains. Phytopathology 97:1127-1185.

Lim, Y.W., J.J. Kim, M. Lu and C. Breuil. 2005 Determining fungal diversity on *Dendroctonus ponderosae* and *Ips pini* affecting lodgepole pine using cultural and molecular methods. Fungal Diversity 19:79-84.

Okada, G., K. Jacobs, T. Kirisits, G. Louis-Seize, K.A. Seifert, T. Sugita, A. Takematsu, M.J. Wingfield. 2000. Epitypification of Graphium penicilliodes Corda, with comments on the phylogeny and taxonomy of Graphium-like synnematous fungi." Studies in Mycology 45: 169-188

Uziel, A. and Kenneth, R.G. 1999. Mycopathologia. Influence of commercially derived lipids and a surfactant on the mode of germination and process of germ-tube formation in primary conidia of two species of Erynia subgenus Neopandora (Zygomycotina: Entomophthorales). 144(3):153-63.

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